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PHYSICAL CHEMISTRY OF HYALURONIC ACID[1,2]

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In studying the physicochemical characteristics of a polymer-type macromolecule, a constituent of solid tissues, the basic questions are: *1)* What changes, if any, has the molecule undergone in the course of the preparation procedure? *2)* How representative is the purified sample of the macromolecules of the tissue? Since hyaluronic acid is a building block of connective tissue, these points must be considered in studying its molecular parameters.

The two questions propounded cannot be answered at the outset of such a study, and therefore, certain assumptions must be made. One is that the purified polysaccharide, free of all proteins, is the molecular entity on which physicochemical studies should be carried out. Another assumption is that by removing all of the proteins one may break up a naturally-occurring molecular complex, the physicochemical characterization of which is of primary importance. The former stresses the purification process and is less concerned with possible degradation in the course of preparation with the yield of the method. The latter emphasizes the importance of a mild preparation method and disregards the consideration that physicochemical studies should, preferably, be made only on molecular entities which have been chemically well characterized. Both lines of thought are well represented in the literature of the last ten years on the physical chemistry of hyaluronic acid.

Since hyaluronic acid can be prepared free of proteins, with a fairly high degree of polymerization retained, this discussion will deal with the physical chemistry of this polysaccharide. The purity of the hyaluronic acid preparations used in the studies reported in this paper was tested by hexosamine, hexuronic acid and nitrogen determinations. It was found that when the hexosamine-hexuronic acid molar ratio is close to one and the nitrogen content is not much higher than would be expected from the hexosamine content, it is assumed that the preparation is protein-free. Preparations with a protein content of up to 5% were often used in physicochemical studies. This protein content was calculated on the assumption that all the hexosamine nitrogen represents pro-

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[2] Paper 70, Retina Foundation.

tain 16% nitrogen. Sulfur and galactosamine erminations were also used to check the pres- of sulfated polysaccharides. Several physico- mical investigations were reported on parations which contained up to 7% of un- ntified sulfated polysaccharides. Many more e never checked for the presence of this sible impurity.

The significance of the presence of proteins n amount of less than 5%, from the view- t of molecular configuration, is unknown. re is no way to tell, at this time, what the l nitrogen content in some preparations icates. It may result from incomplete hy- lysis before hexosamine determination or n other errors in hexosamine analysis. It originate from the presence of bacteria or n proteins completely unrelated to the poly- charide. On the other hand, it may represent peptide bridges cross-linking the polysac- ide chains.

ur present knowledge about the physical mistry of hyaluronic acid will be reviewed in iscussion of such molecular parameters as ght, dimensions, shape, volume and hydra- The charge effect on the structure of this ic polymer and its interactions will be men- d briefly.

MOLECULAR WEIGHT

he molecular weight of hyaluronic acid is in the range of 50,000 to 8×10^6, depending on the source and on the methods of preparation and determination. The lowest polymer is that prepared from the bovine vitreous body, which gives a weight average molecular weight of less than 1×10^6. Hyaluronic acid prepared from bovine synovial fluid, human rheumatoid arthritic synovial fluid, human umbilical cord, pig skin and rooster comb exhibits a weight average molecular weight of well over 1×10^6.

Whether or not the hyaluronic acid prepared represents the average molecule of the tissue depends greatly on the yield of the method of preparation and on how well the total molecular population is represented in the sample. In order to evaluate the hyaluronic acid preparation from this aspect, one must know its concentration in the tissue. Such an evaluation can be made only on the bovine vitreous body because of lack of data on other tissues.

Molecular weight determinations on hyaluronic acid prepared from the bovine vitreous body have been reported by various authors, using the methods of osmotic pressure, sedimentation-diffusion and light scattering (table 1). The methods of preparation used to obtain pure hyaluronic acid from various tissues can be divided into two types: One is the removal of the proteins present in the tissue or in the tissue extract by means of proteolytic enzyme treatment, by selective precipitation or by extraction methods. The other is based on the fact that the electrophoretic mobility of hyaluronic acid is

TABLE 1. MOLECULAR WEIGHT OF HYALURONIC ACID PREPARED FROM BOVINE VITREOUS BODY

Methods of Preparation	Approx. Yield %	Molecular Weight × 10^{-3}			Reference
		Osmotic Pressure ($\bar{M}_n$)*	Sedimentation, Diffusion ($\bar{M}_w$)†	Light Scattering ($\bar{M}_w$)†	
oval of proteins by loroform extraction	20–30		245	500	(10)
d rosin and NaCl precipitation	50–60		87	340–500	(4, 8, 13)
			(13)	(4, 8)	
lene sulfonate precipitation	20–30		296	1270	(10, 11)
			(10)	(11)	
zymatic hydrolysis					
epsin-trypsin, trichloro-acetic acid precipitation	100		57		(10)
papain, cetylpyridinium ancreas extract, trichloro-acetic acid precipitation	100	270		300	(4, 8) (35)
oval of hyaluronic acid					
ctrophoresis			178		(12)
ctrodeposition			220	680	(10)

* = number average molecular weight. † $\bar{M}_w$ = weight average molecular weight.

greater than that of most proteins. Separation of hyaluronic acid from the vitreous body and from synovial fluid by electrophoresis was first reported by Blix (1). However, he did not use electrophoretically prepared hyaluronic acid for physicochemical studies. Varga and Gergely (2) prepared hyaluronic acid from the bovine vitreous body, and this preparation was used for extensive physicochemical studies. The advantage of the electrophoretic separation method is that, most likely, the distribution of the molecules is the same in the purified fraction as in the tissue extract. The disadvantage of this method is that hyaluronic acid cannot be separated from sulfated mucopolysaccharides, which have a greater electrophoretic mobility than hyaluronic acid itself.

A new separation method, called electrodeposition, has been described recently by Roseman and Watson (3). This method is also based on electrophoretic mobility, but uses electrodialysis machines and collects the hyaluronic acid which accumulates as a paste on the dialysis membrane toward the anode. The advantages of this method over electrophoretic separation are that large volumes can be handled and the sulfated mucopolysaccharides can be separated from hyaluronic acid. In most tissue extracts this method alone does not produce protein-free hyaluronic acid. Treatment with Celite was recommended in those cases to remove the last 20–30% of proteins from the sample.

The highest yield (approximately 100%) is obtained when the proteins are removed by pepsin hydrolysis. However, if pepsin is used, incubation at 37° in 0.1 N HCl is required, and this treatment partially depolymerizes the polysaccharide, although polymers small enough to be dialyzable are not produced. Digestion with papain or with crude extracts made from pancreas and intestine does not result in a protein-free hyaluronic acid. However, in combination with phenol, cetylpyridinium or trichloro-acetic acid treatment, the proteolytic enzyme digestion methods will result in preparations with a protein content of less than 5% (4–6).

Other methods have been described for the preparation of hyaluronic acid free from proteins, using chloroform to extract the proteins (7), or using acid resin (8) or xylene sulfonate (9) to precipitate them. All of these methods, however, result in a yield of only 30–60%. Molecular weight determinations indicate that with these different methods of preparation one either degrades the hyaluronic acid in va degrees or one obtains fractions having va molecular weights.

The lowest molecular weight sample o aluronic acid (57–70 × 10^3) was prepared the bovine vitreous body by the pepsin-tr digestion method. This preparation wa polydisperse that the light-scattering mea ments could not be used for molecular w calculations (10). The acid resin precipit method also gave a sample of low molec weight (87 × 10^3), calculated from sedim tion and diffusion data. Using this meth purification, approximately 50% of the aluronic acid precipitates with the pr during acidification and deionization (8). possible that a higher polymer fraction o aluronic acid is removed with the precip Chloroform extraction and xylene sulf precipitation of the proteins result in a l polymer hhaluronic acid fraction (10, 11). molecular weight of hyaluronic acid pre by the electrophoresis or the electrodepo method is within the values mentioned p ously (136–240 × 10^3) (10, 12).

It is interesting to note that the mole weights determined by light-scattering mea ments are always higher than those deter by sedimentation or diffusion studies, probably because the light-scattering m is more sensitive to the polydispersity o sample (8, 10).

A comparison of the molecular weig hyaluronic acid prepared from bovine sy fluid, human rheumatoid arthritic sy fluid, human umbilical cord and rooster suggests that there is no significant diff in the size of the molecules obtained b ferent preparation methods (table 2). M the variation in the molecular weight, calc from sedimentation and diffusion stud within the error of the determination. Th sample which showed a significantly molecular weight was that prepared fro rooster comb (10). Some preparations happened to be studied only by light sca showed a much greater variation (table 2 not yet possible to determine whether th here summarized indicate that hyaluron is present in various tissues in different of polymerization or whether the diff were artificially introduced during prepa The great discrepancy sometimes found b the molecular weight calculated from

BLE 2. MOLECULAR WEIGHT OF HYALURONIC ACID PREPARED FROM VARIOUS CONNECTIVE TISSUES

Source	Method of Preparation	Molecular Weight × 10⁻⁶		Reference
		Sedimentation, diffusion	Light scattering	
vine synovial fluid	Papain, cetylpyridinium		1.6	(8)
	Ultrafiltration, papain	2.0		(14)
	Electrodeposition	1.57	1.8	(10)
man synovial fluid (rheumatoid arthritis)	Electrodeposition	1.86	3.2	(10)
man umbilical cord	Pepsin-trypsin		8.0	(20)
	Papain, cetylpyridinium		5.8	(8)
	Chloroform		3.4	(16)
	Proteolytic enzymes, phenol, cetylpyridinium		3.0	(8)
	Electrodeposition	1.44	2.7	(10)
oster comb	Papain, cetylpyridinium		1.3	(8)
	Electrodeposition	5.13	1.3–4.2	(10)

ntation-diffusion studies and that calculated m light scattering suggests that each prepara- n may have a different degree of polydis- rsity.

DIMENSIONS, SHAPE, VOLUME, HYDRATION

The conclusions drawn about the dimensions d shape of the hyaluronic acid molecule atly depend on the method used for its study. pen the molecule is exposed to a shearing ce in the course of measurement, as in the asurement of double refraction of flow, a formation and uncoiling will occur. This formation and uncoiling is more pronounced the high molecular weight hyaluronic acid an in the low polymer prepared from the reous body (7, 9–11).

The calculated dimensions of the molecule pend greatly on the shape assigned to it. The al ratio (l/d) and the length calculated from based on viscosity, sedimentation, diffusion, uble refraction of flow and light-scattering dies on hyaluronic acid prepared by various thods from different sources, are tabulated in les 3 and 4. The length of the hyaluronic acid lecule prepared from the vitreous body is ween 1600 and 3400 A, depending upon the thod of preparation used. The lowest figure s observed in hyaluronic acid prepared by sin-trypsin digestion. This preparation also l the lowest molecular weight (57,000), and length is that of an almost completely tched molecular chain. Blix and Snellman in ir viscosity and double refraction of flow dies derived a molecular length of 1000–4800 A, assuming that the molecule is a stretched polysaccharide chain (7). Both light scattering and double refraction of flow experiments, assuming a prolate ellipsoid of revolution or a randomly-kinked coil, gave shorter lengths and indicated that the molecular chain is not stretched (2, 4, 8, 10–13).

In the hyaluronic acid samples of higher molecular weight (table 4) the axial ratio calculated from sedimentation-diffusion studies is quite large (700–2600). This dissymmetry is based on the frictional ratio, assuming that the molecule is not hydrated. The dissymmetry calculated from viscosity is somewhat lower, but it also involves the assumption that there is no hydration. The ellipticity of a prolate ellipsoid of revolution, calculated from sedimentation and viscosity data by Blumberg and Ogston (14), was found to be 7.7. From light-scattering and viscosity measurements other authors (10) found an even lower dissymmetry in the high molecular weight hyaluronic acid preparations. All this indicates that the axial ratio of the large hydrated molecules is very low, but that of the lower polymers prepared from the vitreous body is considerably higher.

All light-scattering studies indicate that hyaluronic acid is a randomly-kinked coil with more or less polydispersity and stiffness (4, 8, 10, 11, 15, 16). The length of the molecule calculated from the radius of gyration, assuming the above model, varied between 2400 and 6400 A in the different high molecular weight samples (table 4). This variation may be partially due to the polydispersity of the preparations. Laurent (4) tried to correlate the radius of

Method of Preparation	$[\eta]^*$	Molecular Model: Prolate ellipsoid of revolution: Axial ratio (l/d) calculated from: viscosity	Molecular Model: Prolate ellipsoid of revolution: Axial ratio (l/d) calculated from: frictional ratio	Molecular Model: Prolate ellipsoid of revolution: Length in A calculated from: double refraction of flow	Molecular Model: Prolate ellipsoid of revolution: Length in A calculated from: light scattering	Molecular Model: Random coil: Length in A calculated from: double refraction of flow	Molecular Model: Rigid rod†	Reference
Chloroform extraction	200	70				1000		(7)
	460	115				2600		(7)
	880	265				4800		(7)
	700	138	105		2500			(10)
Acid resin and NaCl precipitation	230	70	62		3400			(4, 8, 13)
Xylene sulfonate precipitation	700	138	230	2500	2100			(10, 11)
Pepsin-trypsin, trichloroacetic acid preparation	230	70	64	1605				(10)
Papain, cetylpyridinium	380	95			2100			(4, 8)
Electrophoresis	690	137	145	2080				(2, 12)
Electrodeposition	640	131	180	2080	2500			(10)

* Limiting viscosity number, concentration in grams per cc.
† Stretched polysaccharide chain with estimated width (d) of 12 A.

TABLE 4. MOLECULAR DIMENSIONS OF HYALURONIC ACID PREPARED FROM VARIOUS CONNECTIVE TISSUES

Source	Method of Preparation	$[\eta]^*$	Molecular Model: Prolate Ellipsoid of revolution: Axial ratio (l/d) calculated from: viscosity	Molecular Model: Prolate Ellipsoid of revolution: Axial ratio (l/d) calculated from: frictional ratio	Molecular Model: Prolate Ellipsoid of revolution: Length in A calculated from: double refraction of flow	Molecular Model: Prolate Ellipsoid of revolution: Length in A calculated from: light scattering	Molecular Model: Random coil	Reference
Bovine synovial fluid	Papain, cetylpyridinium	910	170			3900		(8)
	Ultrafiltration, papain	2200	p = 7.7†					(14)
	Electrodeposition	4830	391	700				(10)
Human synovial fluid (rheumatoid arthritis)	Electrodeposition	3230	322	760	10,300	2900		(10)
Human umbilical cord	Pepsin-trypsin					2100 (sphere)		(20)
	Papain, cetylpyridinium	1330	200			6400		(8)
	Chloroform extraction	3360	327			5400		(16)
	Proteolytic enzymes, phenol, cetylpyridinium	2020	245			5000		(8)
	Electrodeposition	2990	310	520	9,180	2450		(10)
Rooster comb	Papain, cetylpyridinium	950	175			3900		(8)
	Electrodeposition	3130	318	2600	18,100	3700		(10)

* Limiting viscosity number, concentration in grams per cc.
† p = ellipticity.

gyration and the length calculated from it with the molecular weight, assuming that the degree of polydispersity was about the same in the different preparations. The correlation indicated a certain degree of stiffness, or, in other words, nonsolvent-draining, randomly-coiled molecul Laurent also calculated the statistical unit the randomly-kinked coil according to Ku

id Kuhn (17) and found it to be 300 A, containing 60 monosaccharide units. This indicates onsiderable stiffness of the glucosidic linkages hich can be explained by several mechanisms, ich as solvent-solute interaction, interaction ith proteins present in small amounts in the reparation, or cross-linkages.

The problem of the hydration of the hyluronic acid molecule is closely connected with ie problem of molecular volume. It is obvious, om many experiments carried out on pure yaluronic acid (10) and on hyaluronic acid reparations containing proteins (18, 19), that he hydrodynamic volume or the hydrodyamically effective volume is many times greater han that calculated from the dry partial specific volume. The effective volume of a random oil can be calculated as an equivalent rotational ellipsoid of the prolate type, which comrises, then, the whole domain of the molecule ncluding the randomly-coiled molecular chain nd the water in between and surrounding it, as ell as whatever additional proteins or other aterials are present within the domain. This ffective hydrodynamic volume was calculated y various methods from light-scattering, sedimentation and viscosity measurements (10). In he case of low polymer hyaluronic acid prepared om the vitreous body by electrodeposition, the ydrodynamic specific volume was approxiately 6, but in the case of large polymers, uch as hyaluronic acid prepared from synovial uid and umbilical cord by the electrodeposition ethod, this value was between 1000 and 1860. ther authors (14, 18–20) found similarly high alues for the hydrodynamic specific volumes both in high molecular weight hyaluronic acid and in hyaluronic acid, containing 20–30% protein, prepared from synovial fluid by ultrafiltration.

This large volume of water in the domain of the hyaluronic acid coil is not bound to the polysaccharide by long-range forces, as suggested by Jacobson and Laurent on the basis of dielectric studies (21). Laurent showed recently (22) that the x-ray diffractogram of water does not change in the presence of 2% hyaluronic acid as one would expect if hydration-water in this amount was bound to the polysaccharide chain.

A new approach to the problem of the solvation and shape of the hyaluronic acid molecule was introduced by Laurent in studying the physical parameters of cetylpyridinium (CP) hyaluronate (umbilical cord) dissolved in methyl alcohol by the light-scattering method (23). These studies were later supplemented by the experiments of Varga, Pietruszkiewicz and Ryan (24) on CP-hyaluronate prepared from the vitreous body and investigated by sedimentation, diffusion and double refraction of flow methods. Table 5 summarizes their results. The molecular weight of CP-hyaluronate increases as expected. The radius of gyration and the length of CP-hyaluronate in methyl alcohol is approximately half that of Na-hyaluronate in water, indicating a coiling up of the chain. The decrease of the viscosity and the dissymmetry supports this finding. The length measured by double refraction of flow increases, suggesting that under the influence of the shearing force the molecule becomes readily uncoiled. All this points to the importance of the solvent-solute

TABLE 5. MOLECULAR PARAMETERS OF CETYLPYRIDINIUM HYALURONATE IN METHYL ALCOHOL

		Light Scattering		[η]*	Sedimentation-Diffusion			Double Refraction of Flow
		M × 10^{-3}	Radius of gyration A		M × 10^{-3}	1/d	Length A f/f₀	Length A
Human umbilical cord†	Na-HA	3.0	2030	2020				
	CP-HA	5.4	1185	670				
	Na-HA (regenerated)	3.0	1800	1360				
Bovine vitreous body‡	Na-HA				178	145	2100	2080
	CP-HA				289	30	900	2400

* Limiting viscosity number, concentration in grams/cc.
† Laurent (23).
‡ Varga, Pietruszkiewicz, and Ryan (24).

interaction and to the effect of charged groups on the molecular configuration of hyaluronic acid.

The molecular and atomic configuration of hyaluronic acid is not well known and both electron microscope and x-ray diffraction studies are inconclusive. Electron microscope studies (11, 25, 26) suggest a filamentous molecular chain. X-ray diffractograms (27) showed four peaks corresponding to 1.3–1.5 A, 2.5 A, 5 A and 10 A interatomic distances. Laurent suggests that the 1.3–1.5 A peak corresponds to carbon-carbon and carbon-oxygen bonds, the 2.5 A peak to the distance of the second neighboring atom, and the 5 and 10 A peaks represent the distance between two glucosidic bonds or that between chains.

CHARGES

The hyaluronic acid molecule is negatively charged when the carboxyl groups on the glucuronic acid moiety are dissociated. Titration studies were made by Jeanloz and Forchielli (28), conductometric and potentiometric titrations by Pantlitschko (29) and titrations at different ionic strengths by Laurent (15). The intrinsic dissociation constant was found by Laurent (15) to be 3.21, which is close to 3.33 (the dissociation constant of glucuronic acid). From electrophoretic mobility data Varga (13) calculated the effective charge of hyaluronic acid at neutral pH. In hyaluronic acid prepared from the vitreous body, of the 216 anionic groups present/molecule only 14 are effective at 0.12 ionic strength, but at 0.02 ionic strength this figure becomes 173, i.e. 80% of the total. The decreased shielding effect of the small ions will influence the shape of the molecule, resulting in uncoiling and possible changes in the solvent-solute interaction which, since we are dealing with a random coil, means changes in the solvent draining through the domain of the molecule. Both the length measured by light scattering (8) and the length calculated from double refraction of flow (11) increase at low ionic strength. Changes in the sedimentation and diffusion properties of hyaluronic acid at low ionic strength can be explained on the same basis (12, 13).

The polyelectrolyte character of hyaluronic acid is clearly shown by its viscous behavior, the dependence of which on ionic strength has been studied by several authors (7, 8, 30, 31). It has also been shown (8) that the viscosity-concentration relationship at various strengths follows the Fuoss equation (32) acteristic of polyelectrolytes.

MOLECULAR INTERACTIONS

The interaction among the hyaluronic molecules in aqueous solution can be obse with all of the physicochemical methods use the characterization of this molecule. The centration dependence of the viscosity (18 33), of the sedimentation and diffusion c cients (10, 12, 13) and of the electropho mobility (13) all indicate intermolecular int tion. The concentration dependence of th tinction angle in double refraction of experiments (2) and the concentration dep ence of the angular dissymmetry of light sc ing at low ionic strength indicate aggregatio the molecules at higher concentration (16).

The shear dependence of the limiting cosity number of various hyaluronic acid arations was studied with both rotating cyl (18, 33, 34) and capillary-type (30) viscom These studies indicate that the deformabili the internal interaction of the hyaluronic molecules is very small.

The solvent-solute interaction in variou aluronic acid preparations has also been st by correlating the molecular weight, the lim viscosity number and the radius of gyrati 10). These studies suggest that the so draining or nonsolvent-draining character hyaluronic acid molecule depends somewh the method used for preparation and possibl the purity of the preparation as well.

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METABOLISM OF HYALURONIC ACID AND CHONDROITINSULFURIC ACIDS[1]

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N INCREASING NUMBER of investigations ve been concerned recently with the metabo- n of acid mucopolysaccharides. Most studies ve employed S^{35} to investigate the *in vivo* tabolism of sulfated polysaccharides. A more ited number of studies have been concerned h *in vivo* turnover rates employing carboxyl cled acetate and labeled glucose as precursors. hile such studies yield considerable informa- n regarding mucopolysaccharide metabolism, e information regarding pathways of bio- thesis is obtained by these techniques.

lthough connective tissues are widely dis- ed throughout the mammal, it is difficult to ain tissues with a sufficient density of con- tive tissue cells to be appropriate for studies biosynthetic pathways. Since the capsular lysaccharide of group A streptococci appears to be identical with mammalian HA[2] (1), this microorganism affords a convenient tool for the study of biosynthesis of HA.

During the past 7 years studies in this laboratory have been concerned with the elucidation of this biosynthetic pathway. It is the purpose of this communication to summarize these studies together with certain evidence concerning the metabolism of acid mucopolysaccharides in mammals. The early studies, conducted in collaboration with Dr. Saul Roseman, were con-

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[2] The following abbreviations are used in this paper: hyaluronic acid, HA; chondroitinsulfuric acid, CSA; uridine diphosphoglucose, UDPG; uridine diphosphoglucuronic acid, UDPGA; uridine diphospho-N-acetylglucosamine, UDPAG; uridine diphospho-N-acetylgalactosamine, UDPAGa; acetylglucosamine-6-phosphate, AG-6-P; uridine triphosphate, UTP; acetylglucosamine-1-phosphate; AG-1-P; adenosine triphosphate, ATP; hyaluronic acid synthesizing system, HASS; cytidine triphosphate, CTP; guanosine triphosphate, GTP; diphosphopyridine nucleotide, DPN.